(19) SPANISH PATENT AND TRADEMARK OFFICE
SPAIN

(11) Publication number: 2 160 495
(21) Application number: 009901235
(51) Int.. Cl. 7: A61K 38/22
A61P 37/02

(12) PATENT OF INVENTION B1

(22) Filing date: 04.06.1999

(43) Publication date of the application: 01.11.2001

Date granted: 30.07.2002

(45) Date grant is announced: 16.09.2002

(45) Publication date of the patent leaflet: 16.09.2002

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(54) Title: Use of VIP and PACAP peptides for the treatment of inflammatory and autoimmune diseases in mammals.

(57) Abstract:
Use of VIP and PACAP peptides for the treatment of inflammatory and autoimmune diseases in mammals.
Use of VIP (Vasoactive intestinal peptide) and PACAP (Pituitary adenylate cyclase-activating peptide) peptides and their fragments and derivatives in the preparation of drugs for the treatment of inflammatory and autoimmune diseases in mammals. These preparations inhibit the production of proinflammatory cytokines and Th1 cell activation, stimulating the activation of Th2 cells.

DESCRIPTION

Use of VIP and PACAP peptides for the treatment of inflammatory and autoimmune diseases in mammals.

State of the art

Inflammatory processes are vital for the survival of all complex organisms. Inflammation is a natural defense process of the body against foreign agents. The accumulation and activation of leukocytes in the site of an aggression is a central event in any inflammatory process (Schaal TJ and Bacon KB; Current Opinion in Immunology 1994, 6:865). An insufficient inflammatory response may compromise the organism's survival, but an excessive response, which may be due to failures in the deactivation mechanisms of the process with different causes, may end up triggering an inflammatory or autoimmune disease (Sacca R et al; Current Opinion in Immunology 1997, 9:851). These diseases are an important cause of morbidity and mortality in mammals due to the tissue damage associated to said processes.

Macrophages play a central role in regulating immune and inflammatory responses. These activities are mediated by a whole series of complex processes involving many macrophage products. As a response to the antigens, and depending on their origin, macrophages secrete proinflammatory cytokines and oxidizing agents, such as TNFa, IL-6, IL-1/BETA, IL-12 and nitric oxide (Laskin DL et al; Annual Review of Pharmacology and Toxicology 1995, 35:655). TNFa and IL-6 are, amongst others, two factors that contribute to the physiopathological changes associated to several states of chronic or acute inflammation. Macrophages also participate in the start, maintenance and control of immune responses, acting as powerful antigen sources, providing a double activation signal for T lymphocytes: the antigen-molecule complex of the main histocompatibility complex (MHC) and a co-stimulating signal mediated by molecules of the B7 family (Lenschow DJ et al; Annual Review of Immunology 1996, 14:233). B7 molecules comprise two isoforms, B7.1 and B7.2, each one Involved in the stimulation of two different types of collaborator T cells (Th), Th1 and Th2, respectively, and each one produces a different set of cytokines (Kuchroo VK et al; Cell 1995, 80:707).

The activation of Th1 cells implies the production of IFN7 and IL-12 factors, amongst others, is associated to the production of antibodies of isotype IgG2a and manifests itself as a delayed inflammatory reaction. The activation of Th2 cells implies the production of IL-4, IL-5 and IL-10, amongst other factors, is associated to the secretion of antibodies of isotype IgG1, inhibits delayed inflammatory response and manifests itself as a humoral response (Constant SL and Bottomly K; Annual Review of

Immunology 1997, 15:297). The factors that determine the differentiation of one or another type of response are mainly the characteristics of the antigen-presenting cells and cytokines present in the microenvironment the response develops in: IL-12 determines the differentiation of Th1 cells whereas IL-4 determines that of Th2 cells. The effect of IL-4 predominates when they are both present (O'Garra AO; Immunity 1998, 8:275). Numerous cases of inflammatory and autoimmune diseases are due to the activation of an inadequate type of Th cells. Diseases such as rheumatoid arthritis, multiple sclerosis, Crohn's disease, graft versus host reaction and others are characterised by an activation of Th1 cells.

The Vasoactive Intestinal Peptide (VIP) is a basic peptide with 28 amino acids the sequence of which is (Mutt V and Said SI; European Biochemistry 1974, 42:581): His - Ser - Asp - Ala - Val - Phe - Thr - Asp - Asn - Tyr - Thr - Arg - Leu - Arg - Lys - Gln - Met - Ala - Val - Lys - Lys - Tyr - Leu - Asn - Ser – Ile - Leu - Asn - NH2.

It was first isolated from the small intestine of pigs and was subsequently identified in the brain and nerve endings of the peripheral nervous system, and determined as a neuropeptide with neuromodulating properties (Fahrenkrug J; Pharmacology and Toxicology 1993, 72:354). It owes its name to its peripheral vasodilating properties. VIP has also been identified in rat mast cells and in granulomas (Cutz E. et al; Nature 1978, 275:661). Immunochemical studies performed in histological sections of thymus, spleen and lymph nodes of rats have identified immuno-reagent VIP in lymphocytes from these organs (Gomariz RP et al; Annals of the New York Academy of Sciences 1992, 650:13; Leceta et al; Advances in Neuroimmunology 1996, 6:29).

VIP exercises its biological effects via membrane receptors belonging to the superfamily of seven hydrophobic domains coupled to G proteins, which transduce the information to the final effective molecules (Laburthe M and Couvineau A; Annals of the New York Academy of Sciences 1988, 527:296). VIP receptors have been characterized in many tissues, such as liver and adipose tissue amongst others, and which correspond to two types, the so-called VIP1 - R (Ishihara T et al; Neuron 1992, 8:811) and VIP2 - R (Lutz E. et al; FEBS Letters 1993, 334:3). Specific receptors for VIP have been characterized in the immune system in a variety of immune cells including peripheral human lymphocytes, human monocytes, rat and mouse lymphocytes, rat alveolar macrophages and rat and mouse peritoneal macrophages (Gomariz RP et al; Biochemical and Biophysical Research Communications 1994, 203:1599; Delgado M et al; Regulatory Peptides 1996, 62:161). VIP modulates a large

variety of immune functions such as the phagocytic function, in each step of the process, the proliferative response, immunoglobulin production, NK activity and cytokine production (De La Fuente M et al; Advances in Neuroimmunology 1996, 6:75).

The pituitary adenylate cyclase-activating peptide (PACAP) is a member of the secretin/VIP/glucagon peptide family for which two molecular forms of are known, PACAP - 38 and PACAP - 27, the sequences of which are, respectively (Ogi K et al; Biochemical and Biophysical Research communication 1993, 196:1511):

PACAP - 38

His - Ser - Asp - Gly - Ile - Phe - Thr - Asp – Ser - Tyr - Ser - Arg - Tyr - Arg - Lys - Gln - Met - Ala - Val - Lys - Lys - Thy - Leu - Ala - Ala – Val - Leu - Gly - Lys - Arg - Tyr - Lys - Gln - Arg - Val - Lys - Asn - Lys - NH2.

PACAP - 27

His - Ser - Asp - Gly - Ile - Phe - Thr - Asp – Ser - Tyr - Ser - Arg - Tyr - Arg - Lys - Gln - Met - Ala - Val - Lys - Lys - Thy - Leu - Ala - Ala – Val - Leu - NH2.

Both peptides are widely distributed in the central and peripheral nervous systems.

There are also PACAP-producing cells in the lungs, pancreatic B cells and intestine (Arimura A; Regulatory Peptides 1992, 37:287). An abundance of positive cells for PACAP in central and peripheral lymphoid cells has been described in the immune system (Gaytan F et al; Cell and Tissue Research 1994, 276:233). Three types of receptors have been described for PACAP (Shivers BD et al; Endocrinology 991, 128:3055; Inagaki N et al; Proceedings of the National Academy of Sciences USA 1994, 91:2679): the type I PACAP receptor (PACAP - R - I) with equal affinity for PACAP - 38 and PACAP - 27, but with a 300 to 1000 times lower affinity for VIP; the type II PACAP receptor (PACAP - R - II), which recognises VIP, PACAP - 38 and PACAP - 27 with the same affinity, and is therefore called common VIP-PACAP receptor and corresponds to VIP receptor VIP1 - R, and the PACAP type III receptor (PACAP - R - III), which corresponds to VIP receptor VIP VIP2 - R. Studies to date on the biological actions of PACAP in the immune system are scarce. The effects of PACAP are in many cases similar to those of VIP, modulating the phagocytic function and proliferative responses.

Description of the invention

The object of this invention is to develop preparations of VIP, PACAP and the like as therapeutic agents in the treatment of inflammatory and autoimmune diseases.

The treatment consists in the administration to mammals that so require an

effective amount of an agent inhibiting the production of tumour necrosis factor (TNF) or of IL-6 in a pharmaceutically acceptable carrier, or the administration to mammals that so require of an effective amount of an agent increasing the production of IL-4, inhibiting the activation of Th1 cells and stimulating the activation of Th2 cells. These agents are VIP, PACAP or some of their active fragments.

The tumour necrosis factor (TNF) is produced by several cell types including monocytes and macrophages, lymphocytes T and B, neutrophils, mast cells, tumour cells and fibroblasts. It is an important regulating factor of other proinflammatory cytokines, such as IL - 1/BETA, IL - 6 and IL - 8. TNFa induces the expression of adhesion molecules in endothelial cells, activates leukocytes to destroy microorganisms, acts upon hepatocytes to increase the synthesis of serum proteins that contribute to the acute phase response and activates the coagulation system. Its overproduction leads to immunopathological diseases, autoimmunity and inflammation.

IL - 6 is a multifunctional cytokine produced both by lymphocytes and by non-lymphoid cells. It regulates several aspects of the immune response, such as the production of proteins mediating the acute phase and hematopoiesis. It also acts as a mediator in inflammatory response. Its production is regulated by several factors, including TNFa, IL - 1 and bacterial endotoxin (LPS).

IL - 4 is a cytokine that inhibits the production of proinflammatory cytokines, promotes the proliferation and differentiation of activated B lymphocytes and increases the expression of type II MHC molecules in B lymphocytes. Its possible clinical use in anti-inflammatory and autoimmune disease treatments has been highlighted.

Proinflammatory cytokine neutralising strategies have been tested in the treatment of inflammatory diseases but the results do not show any long-term improvements. The administration of VIP and PACAP in animal models achieves these effects and our invention consists of the use of a treatment with these neuropeptides in order to reverse pathological inflammatory states and autoimmune diseases.

VIP and PACAP have antiinflammatory effects and inhibit the production of IL - 6 and TNFa. Moreover, VIP and PACAP modulate the ability of antigen-presenting cells to act by inducing the activation, proliferation and differentiation of lymphocytes with a cytokine secretion pattern typical of Th2 cells and conditioning "in vivo" immune responses, favouring the development of responses of the humoral type and inhibiting responses of the cell type.

Description of the drawings

Figure 1 represents the production of TNFa by murine macrophages in culture

($5x10^5$ cells/mL) stimulated with 10 ngr/mL of LPS in the presence or absence of 10^{-8} M of VIP or PACAP during 24 hours.

Figure 2 represents the production of TNFa by murine macrophages in culture ($5x10^5$ cells/mL) after 6 hours in culture with 10 ngr/mL of LPS and to which 10^{-8} M of VIP or PACAP was added at different times.

Figure 3 represents the production of IL - 6 by murine macrophages in culture ($5x10^5$ cells/mL) stimulated with 10 ngr/mL of LPS in the presence or absence of 10^{-8} M of VIP or PACAP during 24 hours.

Figure 4 represents the production of IL - 6 by murine macrophages in culture ($5x10^5$ cells/mL) after 6 hours in culture with 10 ngr/mL of LPS and to which 10^{-8} M of VIP or PACAP was added at different times.

Figure 5 shows the Northern Blot analysis for the presence of TNFa and IL - 6 mRNA in macrophages stimulated with LPS in the presence or absence of VIP or PACAP (18S represents the corresponding rRNA as a control for the total amount of RNA loaded).

Figure 6 represents the survival of mice injected with 400 µgr of LPS and simultaneously or 30 minutes, 1 or 4 hours later with 5 nmol of VIP or PACAP.

A. Control; B: VIP at 0 h.; C: VIP at 0.5 h; D: VIP at 1 h.; E: VIP at 4 h.

Figure 7 represents the number of IL - 4 secreting cells in the spleen and peritoneum detected by means of the enzyme-linked immunoabsorbent spot (ELISPOT) assay in mice immunised in the conditions specified in Example 7 and which simultaneously to the second injection of the antigen received 5 nmol of VIP or PACAP or an injection of saline.

Figure 8 represents the amount of snail anti-haemocyanin immunoglobulins (anti-KLH) of the IgG2a and IgG1 isotypes detectable in serum by means of the enzyme-linked immunoabsorbent assay (ELISA) in mice immunised in the conditions specified in Example 8 and with serum samples taken two weeks after the last injection.

Figure 9 represents the number of IL - 4 producing cells detected by means of the ELISPOT technique in mice that were immunised in the conditions specified in Examples 7 and 8 and which in the second injection received or not 5 nmol of VIP together with 100 µgr of anti-B7.1 or anti-B7.2 IgG.

Embodiment of the invention

The following examples are only to illustrate the results achieved and do not limit the use of the invention detailed in the specified claims.

Example 1

VIP and PACAP inhibit the production of TNFa in macrophages stimulated with LPS

In experiments performed "in vitro" VIP and PACAP inhibit the production of TNFa in murine peritoneal macrophages stimulated with LPS. The greatest degree of inhibition reaches values of close to 60% and occur with stimulation doses of between 1-10 ngr/mL of LPS. The IC50 is of about 80 pM, both for VIP and for PACAP and its effect was observed until the end of the experiment (see Figure 1). The inhibitory effect is the same if both neuropeptides are added up to 1 hour after stimulating the macrophages with LPS, although it decreases gradually until disappearing if added after 4 hours (see Figure 2).

Example 2

VIP and PACAP reduce the circulating levels of TNFa after the injection of LPS

In an experiment performed with mice the circulating levels of TNFa 2 hours after the injection of 25 μgr of LPS were approximately 4 ngr/mL. The simultaneous administration of 5 nmol of VIP or PACAP reduced said levels by 60%.

Example 3

VIP and PACAP inhibit the production of IL - 6 in macrophages stimulated with LPS

In experiments performed "in vitro" VIP and PACAP inhibit the production of IL - 6 in murine peritoneal macrophages stimulated with LPS. The greatest degree of inhibition reaches values of close to 90% and occur with stimulation doses of 10 μgr/mL of LPS. The IC50 is of 8.6 pM, both for VIP and for PACAP and its effect was observed until the end of the experiment (see Figure 3). The inhibitory effect is observed if the neuropeptides are added after stimulation with LPS, although the degree of inhibition is gradually lower (see Figure 4).

Example 4

VIP and PACAP reduce the circulating levels of IL - 6 after the injection of LPS

In an experiment performed with mice the circulating levels of IL - 6 two hours after the injection of 25 μgr of LPS were approximately 1.5 ngr/mL. The simultaneous administration of 5 nmol of VIP or PACAP reduced said levels by 60% and 75%, respectively.

Example 5

VIP and PACAP regulate the production of TNFa and IL - 6 at the transcription level

Mouse macrophages were subjected to the experimental conditions of examples 1 and 3 and their mRNA was isolated and later analysed by Northern Blot to detect TNFa and IL - 6 mRNA. Figure 5 shows the absence of transcripts for TNFa or IL - 6 when the macrophages activated with LPS are also exposed to VIP or PACAP.

Example 6

VIP and PACAP protect from the lethal effects of LPS

An experiment was performed in which survival was studied in mice during a period of 4 days after injecting them 400 µgr of LPS. The results are shown in Figure 6. Mortality in these circumstances was 100% at 36 hours. The simultaneous administration of 5 nmol of VIP or PACAP achieved a survival of 60% at the end of the experiment. The administration of the neuropeptides up to 1 hour after the injection of LPS still registered survival rates close to 50%.

Example 7

VIP and PACAP increase the proportion of IL - 4 secreting cells

Groups of mice were immunised with 50 µgr of KLH emulsified in an adjuvant, the injection being repeated with 100 µgr of KLH two weeks later and simultaneously injecting 5 nmol/mouse of VIP, PACAP or saline solution. Spleen and peritoneum cell suspensions were performed two weeks after the last injection that were cultured for 24 hours in the presence of 50 µgr/mL of KLH, after which the number of IL - 4 producing cells was determined by ELISPOT. In the mice injected with VIP or PACAP the number IL - 4 producing cells increased in the order of 20 times with respect to those that were not treated with these neuropeptides (see Figure 7).

Example 8

VIP and PACAP induce the production of isotype IgG1 antibodies

Groups of mice were immunised with 50 µgr of KLH emulsified in an adjuvant, the injection being repeated with 100 µgr of KLH two weeks later and simultaneously injecting 5 nmol/mouse of VIP, PACAP or saline solution. The levels of anti-KLH and its isotype were determined two weeks after the last injection by IgG1 and IgG2a-specific ELISA. In the mice injected with VIP or PACAP the anti-KLH antibodies detectable in serum two weeks after the last injection were only for the IgG1 isotype, whereas in those receiving the saline solution they were of the IgG2a isotype (see Figure 8).

Example 9

The increase in the proportion of IL-4 producing cells mediated by VIP and PACAP is related with the expression of B7.2 induced by both neuropeptides

Groups of mice were immunised in the same conditions of Examples 7 and 8, but at the time of the second immunisation with KLH the mice that were simultaneously injected with VIP or PACAP received at the same time 100 µgr anti-B7.1 or anti-B7.2 antibody or the same amount of IgG as a control. In the mice receiving anti-B7.2

antibodies simultaneously to the administration of the neuropeptides, the number of IL - 4 producing cells decreased to the proportion reached in the animals that were not injected with the neuropeptides (see Figure 9).

CLAIMS

1. The use of the vasoactive intestinal peptide (VIP) or some of its fragments or a similar product derived for preparing a drug intended for the treatment of inflammatory or autoimmune pathologies characterised by the activation of Th1 cells, such as rheumatoid arthritis, multiple sclerosis, Crohn's disease, graft versus host reaction and others, due to its capacity as an inhibitory agent for Th1 cells and proinflammatory cytokines.

2. The use of the pituitary adenylate cyclase-activating peptide (PACAP) or some of its fragments or a similar product derived for preparing a drug intended for the treatment of inflammatory or autoimmune pathologies characterised by the activation of Th1 cells, such as rheumatoid arthritis, multiple sclerosis, Crohn's disease, graft versus host reaction and others, due to its capacity as an inhibitory agent for Th- cells and proinflammatory cytokines.

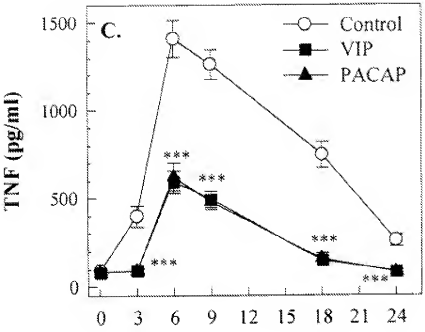

Figure 1

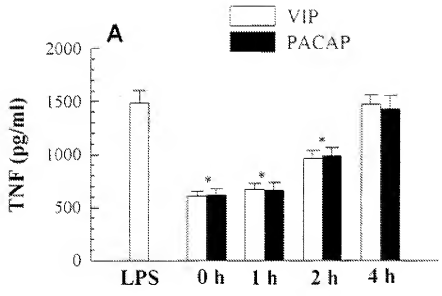

Figure 2

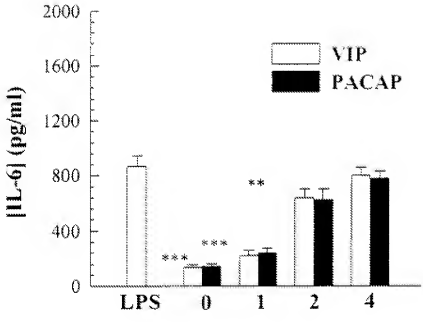

Figure 3

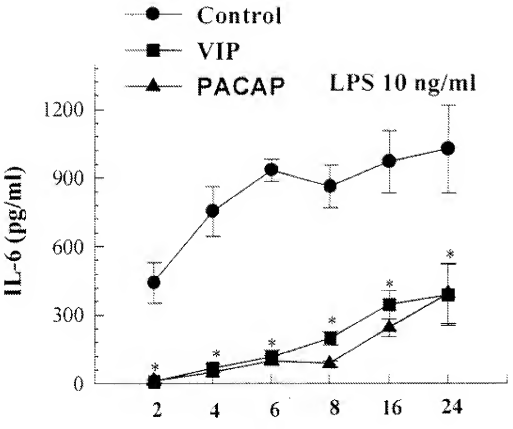
Control
VIP
PACAP
LPS 10 ng/ml
IL-6 (pg/ml)
1200
900
600
300
0
2
4
6
8
16
24
*

Figure 4

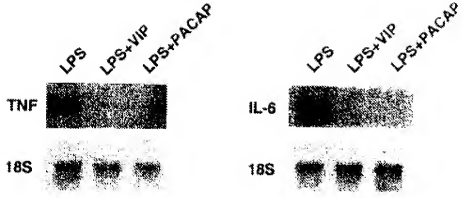

Figure 5

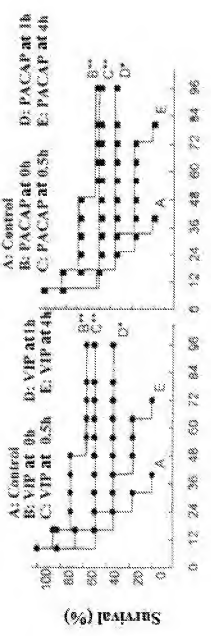
A: Control
B: VIP at 0h
C: VIP at 0.5h
D: VIP at 1h
E: VIP at 4h
A: Control
B: PACAP at 0h
C: PACAP at 0.5h
D: PACAP at 1h
E: PACAP at 4h
Survival (%)
100
80
60
40
20
0
0 12 24 36 48 60 72 84 96
0 12 24 36 48 60 72 84 96
A
E
D*
C**
B**
A
E
D*
C**
B**

Figure 6

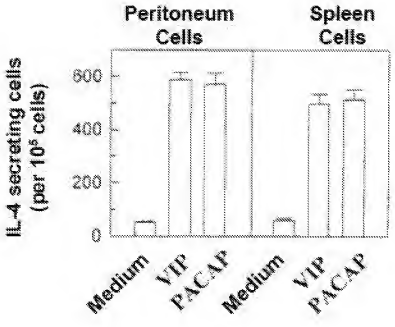
Peritoneum Cells
Spleen Cells
IL-4 secreting cells (per 10^5 cells)
600
400
200
0
Medium
VIP
PACAP
Medium
VIP
PACAP

Figure 7

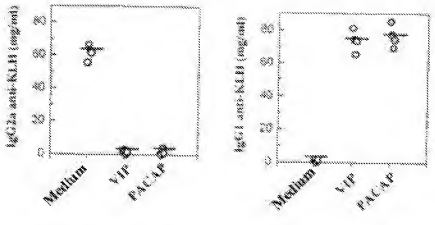
IgG2a anti-KLH (mg/ml)
0
10
20
30
40
Medium
VIP
PACAP
IgG1 anti-KLH (mg/ml)
0
20
40
60
80
Medium
VIP
PACAP

Figure 8

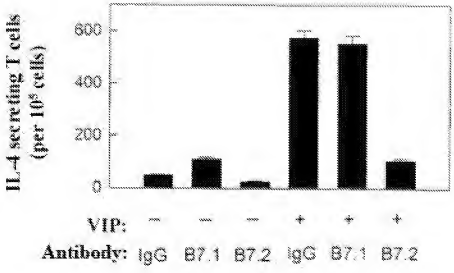
IL-4 secreting T cells
(per 10^5 cells)
600
400
200
0
VIP: – – – + + +
Antibody: IgG B7.1 B7.2 IgG B7.1 B7.2

Figure 9

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VERIFICATION OF TRANSLATION

I, Patricia Koch Moreno of Herrero & Asociados, S.L., Alcalá 35, 28014 Madrid, Spain, hereby declare that I am conversant with the Spanish and English languages and that I am the translator of the document attached and certify that to the best of my knowledge and belief the following is a true and correct English translation of the document (Spanish Patent Application Nº 2 160 495).

Dated this 6th day of May 2008

Patricia Koch Moreno

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